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Expression and cell membrane localization of rat M3 muscarinic acetylcholine receptor produced in Sf 9 insect cells using the baculovirus system

Subhash Vasudevan, Helmut Reiländer, Gabi Maul and Hartmut Michel

Abteilung Molekulare Membranbiologie, Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7, 6000 Frankfurt/M 71, Germany

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A recombinant baculovirus bearing the cDNA coding for the rat muscarinic acetylcholine receptor subtype M3 placed under the control of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene promoter, was constructed. Polymerase chain reaction screening was used to identify the recombinant baculovirus. Northern blot analysis of total RNA from insect cells infected with the recombinant baculovirus indicated that the transcripts were abundant. Binding assays carried out with the muscarinic antagonist [^{3}H]quinuclidinyl benzilate indicated that more than 1×10^6 receptors were produced per cell. Immunofluorescence microscopy showed that the receptor is located on the cell surface.

Muscarinic receptor; Baculovirus expression; PCR screening; Immunofluorescence

1. INTRODUCTION

The class of receptors coupled to G proteins (GTP binding proteins) are growing in number, and their pharmacological relevance leads to increasing interest in their 3-dimensional structure. However, at present there is a vacuum in our understanding of the tertiary structure of G protein-linked receptors [1]. The current consensus arising from structural studies of bacteriorhodopsin [2] and from topography studies of β-adrenergic receptor [3] is that the class of G protein-linked receptors traverse the membrane seven times with connecting intracellular and extracellular loops.

The family of muscarinic receptors where five subtypes have been identified by gene cloning and sequencing, mediate a variety of important physiological events. These subtypes have been broadly classified into two groups based on their G protein and effector coupling characteristics (reviewed in [4]). From the viewpoint of crystallization, the muscarinic acetylcholine receptor may have a unique favourable feature in that the third cytoplasmic loop which connects the putative transmembrane helices 5 and 6, is very large. A similarity with the photosynthetic reaction centre from *Rhodopseudomonas viridis*, which is the first integral membrane protein to yield well-ordered crystals [5] is that the cytochrome and the H-subunit form large extramembraneous parts.

Since there are no rich natural sources of a homogeneous subtype of the muscarinic receptor, we have sought to produce the protein heterologously in insect cells using the baculovirus system (reviewed in [6,7]). In this report we describe the construction of a baculovirus transfer vector containing the cDNA coding for the rat muscarinic receptor subtype 3 (RM3), and further show that the receptor is produced and located on the cell surface of Sf9 cells infected with recombinant baculovirus.

Abbreviations: QNB, quinuclidinyl benzilate; RM3, rat muscarinic acetylcholine receptor subtype 3; Sf 9, *Spodoptera frugiperda* cells; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate

Correspondence address: S. Vasudevan, Abteilung Molekulare Membranbiologie, Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7, 6000 Frankfurt/M 71, Germany. Fax: (45) (69) 6704423.

2. MATERIALS AND METHODS

2.1. *Materials*

Wild-type AcMNPV and baculovirus transfer vectors (pVL1392 and pVL1393) were kindly provided by Max Summers of the Texas A&M University. The plasmid pBluescript KS+ was purchased from Stratagene. Restriction endonucleases and DNA modifying enzymes were from Boehringer Mannheim or Bethesda Research Laboratories. The thermostable DNA polymerase, AmpliTaq was from Perkin Elmer-Cetus. Tissue culture flasks, multidish 6 and related material were from Nunc, Denmark. Spinner flasks for suspension culture were from Techne, UK. Components for TNM-FH medium were purchased from Sigma. Fetal calf serum and gentamycin were from Gibco BRL. Monoclonal antibody M35 was purchased from Chemunex, France. Pluronic F-68 was a gift from BASF, Mannheim.

2.2. *Methods*

2.2.1. Cells and viruses

Sf 9 cells (ATCC no. 1711) were routinely propagated at 27°C in TNM-FH medium supplemented with 5% fetal calf serum and

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50 μg/ml of gentamycin (+0.2% Pluronic F-68, for suspension cultures). Procedures for cell culture, co-transfection, viral infection and isolation of viral genomic DNA were carried out as described [8]. For analysis of RM3, Sf 9 cells were infected with the recombinant virus at a MOI (multiplicity of infection) of 10.

2.2.2. Construction of recombinant vectors

Standard methods for in vitro DNA manipulations were essentially as described [9]. An ~3.8 kb cDNA fragment which contained the open reading frame for RM3 [10] in plasmid pSP65 was generously provided by Peter Seeburg, ZMBH, Heidelberg. The 5'- and 3'-untranslated sequences were removed by the following strategy. Two oligo-nucleotide primers (26-mer, 5'-AATCTAGAATGACC-TTGCACAGTAAC-3' and 21-mer 5'-CCCCCGAGAGGGTCGC-TGCAG-3') were used to amplify a 200 bp fragment by PCR [11]. The amplified fragment was sequentially precipitated, digested with the restriction endonuclease *Xba*I, isolated from an 1.2% agarose gel and ligated to plasmid pBluescript KS+ that was previously linearized by cutting with *Xba*I and *Sma*I. The resultant plasmid was digested with *Sty*I (+92) and *Eco*RV before ligation to a 1717 bp *Sty*I-*Tha*I fragment from the parent plasmid. The resultant plasmid pBSKSMac3 was the source of cDNA for the construction of the transfer vectors pVL Mac3 and pVL Cam3 (Fig. 1). The correctness of the constructs was confirmed by restriction analysis and DNA sequencing.

2.2.3. Isolation of recombinant baculovirus

The transfer vectors pVL Mac3 and pVL Cam3 (10 μg) were co-transfected separately with AcMNPV genomic DNA (1 μg) to generate the recombinant baculovirus. Visually screened plaques were analysed by PCR screening to identify the recombinant virus. The PCR primers (BacF, 5'-AATGATAACCATCTCGCAATAAA-TAAG-3' and BacR, 5'-CTTTGAACACCAGGGAAACTTCA-AGGAG-3') are designed such that they can be used with all currently available transfer vectors based on the polyhedrin promoter. Briefly, plaques that were picked by visual screening were eluted into cell culture medium (1 ml) and used to infect ~1 × 10^6 cells in Multidish 6. After 2 h incubation at 27°C a further 1 ml medium was added and incubation continued for 6–8 days. The supernatant was collected after centrifugation at 4000 rpm for 10 min. After saving a portion of the supernatant as master viral stock, the remainder (1 ml) was centrifuged at 100000 × *g* for 30 min in a TL-100 ultracentrifuge (Beckman). The viral pellet was suspended in 200 μl of STET buffer (8% sucrose, 0.1% Triton X-100, 1 mM EDTA, 50 mM Tris-HCl, pH 8), and boiled for 1 min. The suspension was extracted successively with phenol, phenol/chloroform, chloroform, and finally precipitated with ethanol. The DNA was dissolved in 30 μl of water and a portion (10 μl) was used for PCR.

2.2.5. Other methods

Northern analysis was carried out as described [9]. [^{3}H]Quinucli-

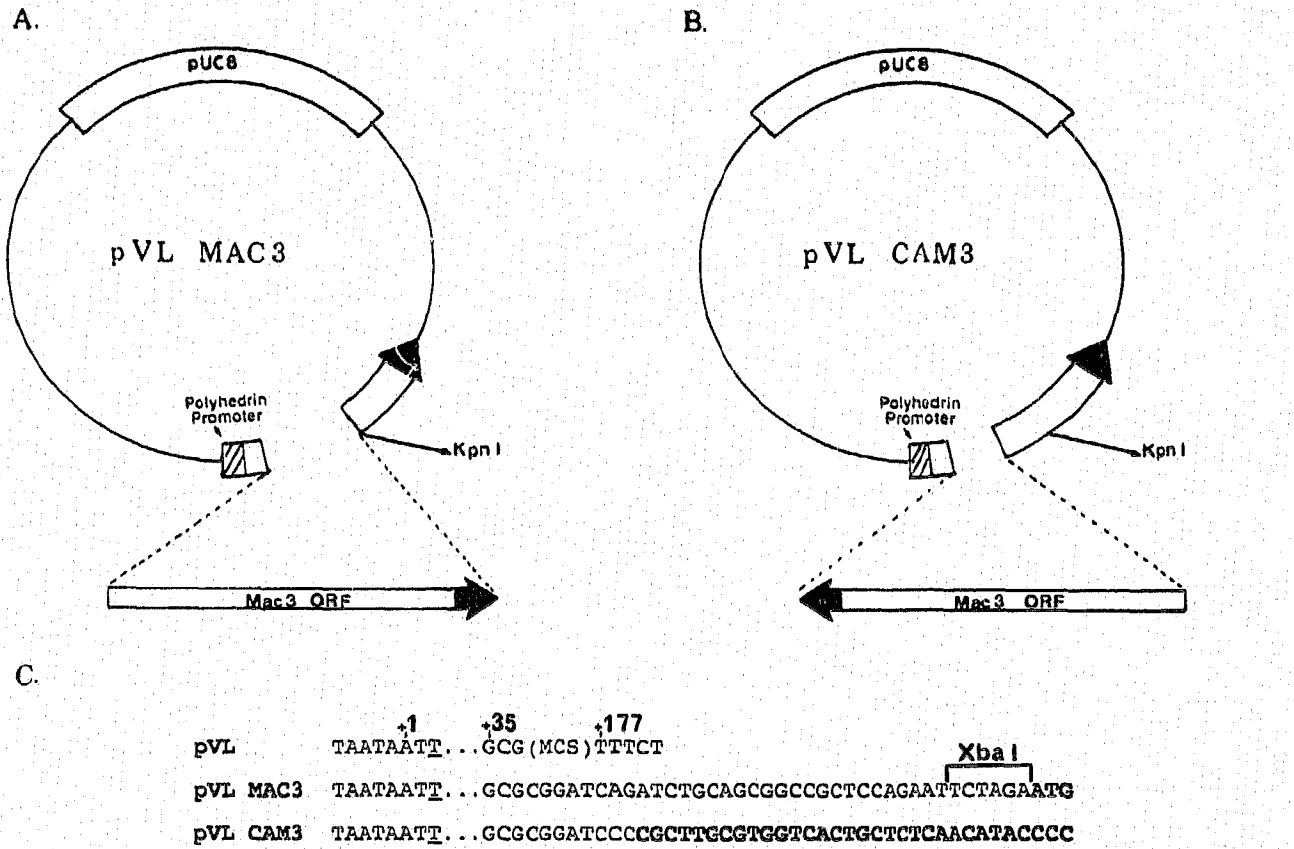

Fig. 1. Structure of recombinant baculovirus transfer vectors pVL MAC3 and pVL CAM3. (A) pVL MAC3 was obtained by inserting an ~1.85 kb *Xba*I-*Kpn*I fragment (derived from pBSKSMac3) containing the entire 1.77 kb RM3 coding sequence into pVL 1392. (B) pVL CAM3 was obtained as follows: the plasmid pBSKSMac3 was cut with *Kpn*I followed by treatment with T4 DNA polymerase to produce a blunt end and digested with the *Xba*I to exise an ~1.85 kb fragment which was inserted between *Sma*I and *Xba*I sites of pVL 1393. (C) The positions of nucleotides around the mutated translation initiation codon of polyhedrin gene are indicated. The sequence between +4 and +34 of the polyhedrin gene and the multiple cloning sites (MCS) inserted between +38 and +177 are not shown. The sequence of the ATG start codon of RM3 in pVL MAC3 and the 3'-untranslated region of RM3 in pVL CAM3 are indicated in bold.

dinyl benzilate (QNB) binding assays on whole cells and membrane preparations were carried out as described [12]. Analysis of binding data was carried out using standard computer programs. Immunofluorescence using monoclonal antibody M35 was carried out essentially as described [13].

3. RESULTS AND DISCUSSION

The strategy employed for the construction of recombinant transfer vectors pVL Mac3 and pVL Cam3 took into account some of the salient features required for expression of heterologous genes in eukaryotic cells. The presence of an upstream initiation codon without an in-frame termination codon before the start site for translation initiation of RM3 gene may be detrimental [10,14]. Also it is known that GC rich 5' untranslated regions, and the presence of secondary structures near the translation initiation region of mRNAs can have profound effect on expression. To circumvent these problems, the 5'-untranslated region of RM3 cDNA was removed by PCR with the simultaneous introduction of an upstream *Xba*I site to facilitate cloning into the transfer vector. A convenient *Tha*I site 34 bp downstream from the RM3 gene termination codon allowed the removal of the 3'-untranslated region which has been known to reduce mRNA stability [14]. The choice of the transfer vector was based on the observation that the retention of coding sequences for the initial amino acids of the polyhedrin gene resulted in higher levels of the heterologously produced protein [15]. Fig. 1 summarises the construction of the recombinant vectors.

Homologous recombination that results in the formation of recombinant baculovirus is a rare event [7]. Hence, the wide applications of PCR technology prompted the design of the two primers for the screening of viral DNA obtained from visually screened plaques. The procedure for isolation of viral DNA suitable for PCR screening was developed to allow large numbers of plaques to be analysed simultaneously. When a plaque had a mixture of wild-type and recombinant virus, the PCR amplification revealed a product of about 0.7 kb (polyhedrin DNA) in addition to a band of the expected size for the cloned DNA (e.g. Fig. 2, lane 5). The purity of the recombinant baculovirus was indicated by the complete absence of the 0.7 kb fragment. There are several advantages in using

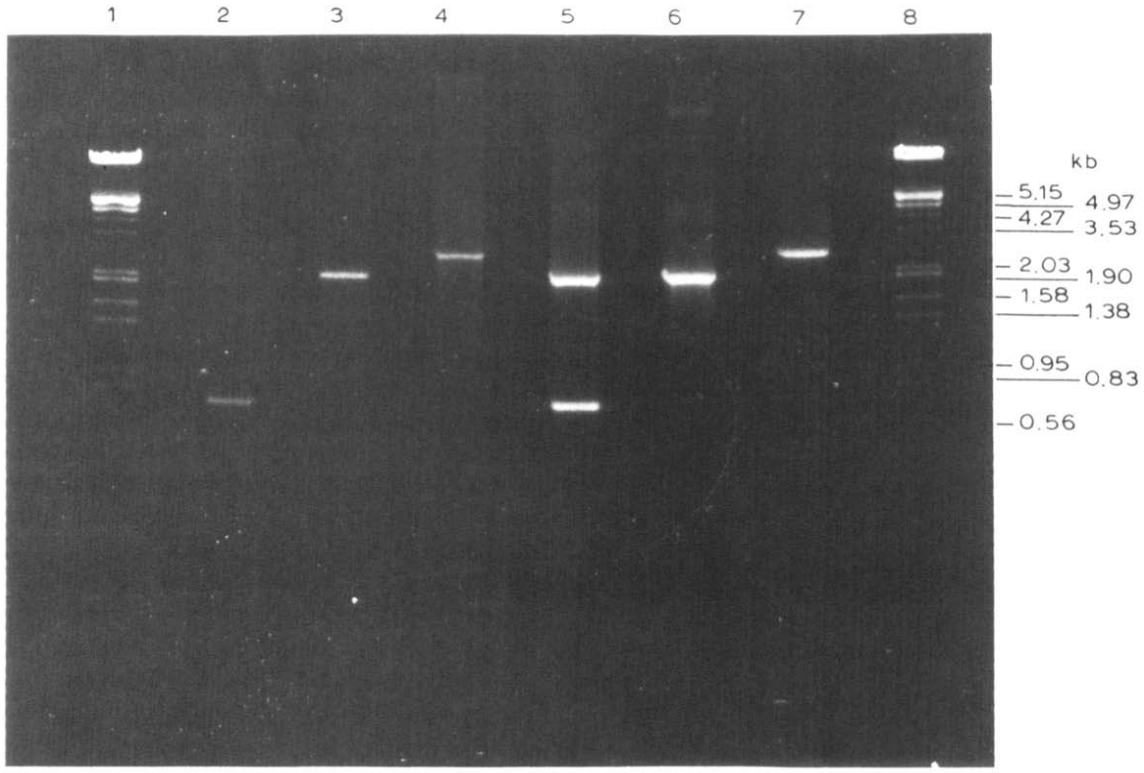

Fig. 2. PCR amplifications with wild-type and recombinant baculovirus DNA, and with recombinant transfer vectors. The DNA amplifications were carried out in 100 μl reaction volume containing 0.26 pmol of each primer, 200 μM of each dNTP and 10 μl of template in a buffer that contained 10 mM Tris-HCl, pH 8.4, 2 mM $MgCl_2$, 20 mM KCl and 0.1 mg gelatin, with 2–3 units of AmpliTaq DNA polymerase. The samples were amplified for 25 cycles with DNA denaturation at 93°C for 1.5 min, primer annealing at 60°C for 2 min and primer extension at 72°C for 4 min. (Lanes 1 and 8) Size markers; (lane 2) 0.7 kb fragment amplified from wild-type baculovirus; (lane 3) an ~2.2 kb fragment amplified from pVL MAC3; (lane 4) an ~2.6 kb fragment amplified from pVL CAM3; (lane 5) mixture of two fragments from an apparently occlusion negative plaque; (lane 6) band from an occlusion negative plaque with RM3 gene in correct orientation and (lane 7) wrong orientation with respect to the polyhedrin promoter.

PCR screening to identify a recombinant baculovirus, mainly it obviates the need to show proof for the isolation of a recombinant virus by Southern hybridization with probes from the foreign gene and portion of polyhedrin gene deleted in the recombinant virus. Additionally, PCR screening can identify possible recombination errors especially when the foreign gene is closely related to the genome of Sf 9 cells [16].

Sf 9 cells infected with PCR-screened recombinant baculovirus and appropriate controls were examined for the presence of RM3 transcripts by Northern analysis. As shown in Fig. 3, transcripts were detected when cells were infected with recombinant viruses containing the RM3 gene in the wrong orientation and correct orientation with respect to the polyhedrin promoter. The observed sizes of the transcripts are in good accordance with the calculated sizes when the contributions of the polyhedrin transcriptional start and polyadenylation signals are taken into account. The transcripts could also be discerned by Methylene blue stained Northern blot (results not shown), thus indicating the efficiency of transcription from the polyhedrin promoter.

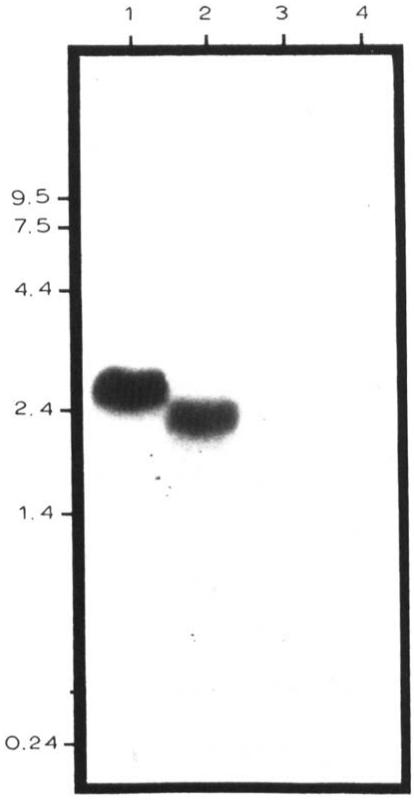

Fig. 3. Northern analysis of total RNA from infected and non-infected Sf 9 cells. Autoradiogram of Northern blot of total RNA isolated from: (1) Sf 9 cells 48 h post-infection with recombinant virus bearing RM3 gene in the wrong orientation; (2) Sf 9 cells 48 h post-infection with recombinant virus where RM3 is in the correct orientation; (3) Sf 9 cells infected with wild-type baculovirus; and (4) from non-infected Sf 9 cells. RNA-sizes are given in kb. Hybridization was carried out with RM3 probe from pBSKSMac3.

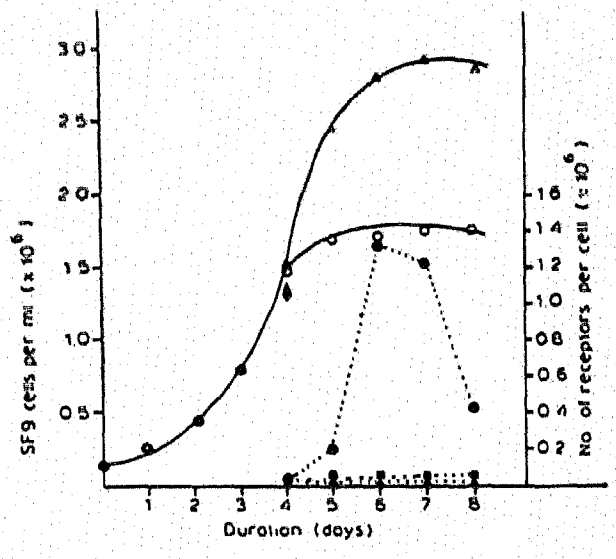

Fig. 4. The growth of non-infected and infected Sf 9 cells related to the expression of RM3 as determined by [^{3}H]QNB binding assays. (Δ—Δ) Growth curve of non-infected Sf 9 cells. (○—○) Growth curve of Sf 9 cells infected with recombinant baculovirus with RM3 in the correct orientation. The arrowhead indicates the point of infection. The specific number of [^{3}H]QNB binding sites are defined as that which can be displaced by 10 μM atropine. The number of [^{3}H]QNB binding sites on non-infected Sf 9 cells (▲---▲), recombinant baculovirus infected cells (■---■), with RM3 in wrong orientation and (●---●), with RM3 in correct orientation were measured at the indicated intervals.

Infection of Sf 9 cells grown in suspension culture with recombinant baculovirus (bearing the RM3 gene in the correct orientation with respect to the polyhedrin promoter) revealed a time-dependent increase in the

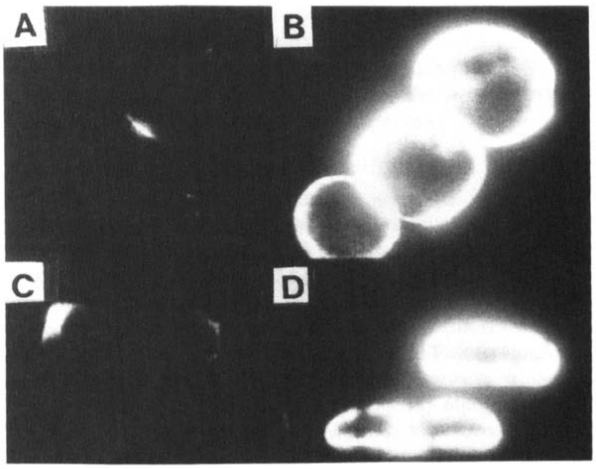

Fig. 5. Fluorescence scanning microscopy of non-infected and recombinant baculovirus infected Sf 9 cells treated with monoclonal antibody M35, and detected by FITC-conjugated goat anti-mouse IgM. (A) Horizontal and (C) vertical views of non-infected Sf 9 cells. (B) Horizontal and (D) vertical views of Sf 9 cells treated 48 h after infection with recombinant baculovirus with RM3 in correct orientation with respect to the polyhedrin promoter.

receptor binding sites on whole cells as determined by [^{3}H]QNB-binding assay (Fig. 4). The level of the receptor was maximal between 48 and 72 h post-infection, indicating more than a million receptors per cell. Membranes prepared from cells harvested 36 h post-infection showed receptor concentrations of 12–18 pmol/mg protein (compare with 0.7 pmol/mg from native sources [17]).

The monoclonal antibody M35 has been shown to be useful for studying the distribution of muscarinic receptors in the human cerebral cortex [18]. The expression of RM3 in insect cells was analysed by fluorescence laser scanning microscopy using the monoclonal antibody M35 and a second FITC tagged antibody. Fig. 5 shows that M35 specifically recognised an epitope on cells infected with the recombinant baculovirus. Since the cells were not permeabilized prior to fixing, we conclude that the receptor is localised to the cell surface. The dark patches observed in both the horizontal and vertical sections give further credence to our conclusion.

Taken together, these results show that we have constructed a recombinant baculovirus that upon infection produces RM3 on the cell surface of Sf 9 cells. The stage is now set for further pharmacological, biochemical and physical analysis of an important member of the G protein-linked class of receptors.

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